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(21) International Application Number: PCT/US90/00115 (22) International Filing Date: 10 January 1990 (10.01.90) (30) Priority data: 295,934 11 January 1989 (11.01.89) US (71) Applicant: THE UNITED STATES OF AMERICA, represented by THE SECRETARY, UNITED STATES DEPARTMENT OF COMMERCE [US/US]; Washington, DC 20231 (US). (72) Inventors: WONDISFORD, Frederic, E. ; 21 Englishman Court, Rockville, MD 20852 (US). WEINTRAUB, Bruce, D. ; 6620 Sulky Lane, Rockville, MD 20852 (US). (74) Agents: OLIFF, James, A. et al.; Oliff & Berridge, 277 S. Washington Street, Alexandria, VA 22320 (US).		(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: BIOLOGICALLY ACTIVE SYNTHETIC THYROTROPIN AND CLONED GENE FOR PRODUCING SAME (57) Abstract Substantially pure recombinant TSH has been prepared from a clone comprising complete nucleotide sequence for the expression of the TSH. Diagnostic and therapeutic applications of the synthetic TSH are described.		

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BIOLOGICALLY ACTIVE SYNTHETIC
THYROTROPIN AND CLONED GENE FOR PRODUCING SAME

The present invention is related generally to the isolation and characterization of new genes and proteins. More particularly, the present invention is related to providing isolated, substantially pure, biologically active human thyrotropin (rTSH) synthesized by a cloned gene.

Thyrotropin (TSH) is a pituitary peptide hormone which regulates important body functions. However, heretofore there was no stable, reliable and economic means of synthesizing this important hormone.

SUMMARY OF THE INVENTION

It is, therefore, an object of the present invention to provide biologically active, synthetic human thyrotropin in substantially pure, isolated form.

It is another object of the present invention to provide a cloned gene which directs the expression of biologically active human thyrotropin in a suitable vector.

It is a further object of the present invention to provide an assay kit for measuring thyroid-stimulating hormone as well as other thyrotropin substances such as thyroid-stimulating immunoglobulins and the like.

It is a further object of the present invention to provide a method of diagnosing and treating human thyroid cancer.

Other objects and advantages will become evident from the following detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other objects, features and many of the attendant advantages of the invention will be better understood upon a reading of the following detailed description when considered in connection with the accompanying drawings wherein:

Figure 1 shows schematic construction of the expression vectors. pSV2.G and pAV2 are pBR322 derived

plasmids with the origin of replication (ori) and ampicillin resistance gene (amp^r) as shown. pSV2.G contains the early promoter of SV40 upstream of the HindIII cloning site, rabbit β -globin cDNA, and poly-adenylation site/intron of SV40. pAV2 has the entire adenovirus-associated virus genome (4.7 kb) with its three promoters P5, P19, P40, and polyadenylation site. The HindIII cloning site is downstream of the P40 promoter. Human TSH and hCG α was inserted into the HindIII site of either plasmid forming pAV2-hTSH β , pAV-hCG α , pSV2.GhTSH β , and pSV2.G-hCG α .

Figure 2 shows Northern blot analysis of transfected 293 and COS cells. Total cellular RNA was separated on a 1% agarose-formaldehyde gel and transferred to a nylon membrane. Forty micrograms of total RNA from a control of transfected cell culture were applied to each lane. Human CG α and hTSH β were abbreviated α and β in construct names and in other figures. Cells that were not transfected are labeled control. Cells transfected with a calcium phosphate precipitate lacking DNA are labeled mock. Lanes 1-4 are total RNAs derived from 293 cells; lanes 5-9 are RNAs derived from COS cells. The migration position of an RNA standard in kilobases and hTSH β mRNA from human pituitary is shown to the left of the autoradiograph. Below the autoradiograph is a simplified version of Figure 1 showing the pAV2 and pSV2.G plasmid as a single line, the promoters as blackened circles, the 2.0 kb hTSH β genomic fragment as a box containing two exons (blackened regions) and known polyadenylation signalsite sequences as open arrowheads. Below each construct, pAV2- β and pSV2.G- β , is the predicted RNA initiating at the specified promoter, and splicing as shown. Solid arrowheads, poly(A) tails. Predicted size in kilobases (kb) is shown to the right of each mRNA species.

Figure 3 shows the results of gel chromatography. Cell medium from 293 cells transfected with

5 pAV2-hCG /pAV2-hTSH β /pVARNA was chromatographed on a Sephadex G-200 fine column. In addition, standard preparations of hTSH, hCG α , and hTSH β (described in the text) were chromatographed on the same column. RIA of human α and TSH β and IRMA for hTSH was done on each 1.5-ml fraction. Elution position of bovine thyroglobulin (void, Vo). BSA [67,700 (67k)] and ovalbumin [45,000 (45k)] is marked, as well as those of the standard preparations of hTSH, hCG α , and hTSH β .

10 Figure 4 shows the results of human TSH IRMA. A highly sensitive and specific hTSH IRMA was performed on two pituitary hTSH standards. World Health Organization 80/558 (WHO STD) and NIH I-6 (I-6 STD), and the medium from 293 cells after transfection with pAV2-hCG , pAV2-hTSH , and pVARNA (pAV2 α / β /pVARNA). A logit transformation of assay binding was plotted vs. arbitrary units of sample volume added to the assay.

15 Figure 5 shows the results of in vitro bioassay of hTSH in rat thyroid cells. The human pituitary TSH standards and medium from transfected 293 cells used in this assay are defined in the legend to Figure 4. This in vitro bioassay measures TSH stimulated ¹²⁵I uptake into rat thyroid cells (FRTL5). Iodide trapping by pituitary standards and medium from a transfected culture are normalized to TSH immunoactivity in an IRMA.

25 DETAILED DESCRIPTION OF THE INVENTION

The above and various other objects and advantages of the present invention are achieved by the cloning of complete nucleotide sequence which directs the synthesis of biologically active human thyrotropin in a suitable expression vector and isolating substantially pure form of the synthesized hormone.

30 Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described

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herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned hereunder are incorporated herein by reference. Unless mentioned
5 otherwise, the techniques employed herein are standard methodologies well known to one of ordinary skill in the art.

The term "substantially pure" as used herein means as pure as can be obtained by employing standard
10 conventional purification techniques known in the art.

The term "biologically active" as used herein means that the recombinant hormone, even though not identical in physical or chemical structure or composition as the naturally occurring hormone, yet is functionally equivalent thereto.
15

MATERIALS AND METHODS

Materials

Restriction and modifying enzymes were obtained from Bethesda Research Laboratories (Gaithersburg, MD) and Pharmacia (Piscataway, NJ). ^{32}P and ^{35}S compounds
20 were purchased from both DuPont New England Nuclear (Boston, MA) and Amersham/Searle Corporation (Arlington Heights, IL). Gene Screen and Gene Screen Plus membranes (New England Nuclear) were used in all DNA and RNA transfer procedures. A transformation competent strain of
25 Escherichia coli. HB101 was obtained from Bethesda Research Laboratories and used in all transformations. Oligonucleotides were purchased from the Midland Certified Reagent Company (Midland, TX). Cloning and
30 propagation of DNA was done in accordance with NIH guidelines. Sephadex G-200 fine and concanavalin A-Sepharose were obtained from Pharmacia Fine Chemicals. α -Methyl glucoside and α -methyl mannoside were purchased from Sigma (St. Louis, MO). Human TSH, hCG α , and hTSH β were
35 provided by the NIDDK National Hormone and Pituitary Program (Bethesda, MD). Protein standards were purchased from sigma or Pierce Chemical Co. (Rockford, IL).

Genomic Screening

Independent recombinant phage clones (1×10^6) of an EMBL3 human genomic leukocyte library were screened for the presence of human TSH β using a radio labeled mouse TSH β cDNA obtained from W. Chin. Brigham and Women's Hospital, Harvard Medical School, Boston, MA, and two separate clones were identified. A 34 base oligonucleotide, with the same sequence as the first 34 bases of the 5'untranslated sequence of bovine TSH β cDNA, was 5'-end labeled with [$t\text{-}^{32}\text{P}$]ATP to specific activity of $5\text{--}8 \times 10^6$ cpm/picomol using polynucleotide kinase; mouse TSH β cDNA was [$\alpha\text{-}^{32}\text{P}$] dCTP labeled with a random primer to a specific activity of $1\text{--}5 \times 10^6$ cpm/ μg . Both were used to probe Southern blots of restriction digests of one clone.

Subcloning and Sequencing

Genomic fragments were subcloned into pUC18 and mp13 to facilitate restriction mapping and sequencing using the dideoxy chain termination method of Sanger (Sanger et al, 1977, Proc Natl Acad Sci USA 74:5463-5467).

Expression Vectors

A 621 bp hCG α cDNA (obtained from J. Fiddes, California Biotechnology Inc., Palo Alto, CA) was inserted downstream of the early promoter of SV40 in pSV2.G [obtained from B. Howard, NIH (Bethesda, MD)] (Gorman et al, 1982, Mol Cell Biol 2:1044-1051) or the P40 promoter of adeno-associated virus in pAV2 (Laughlin et al, 1983, gene 23:65-73) at the HindIII site forming pSV2.G-hCG α and pAV2hCG α (Fig. 1). HindIII Linkers were ligated to a 2.0 kb PvuII fragment of the hTSH β gene containing 277 bp of 5'-intron, both coding exons, a 450 bp intron, and approximately 800 bp of 3'-flanking DNA. It was inserted into the same HindIII sites as hCG α forming pSV2.G-hTSH β and pAV2-hTSH β (Fig. 1). All plasmids were subjected to multiple restriction enzyme digestions to confirm the presence of only one insert in the proper

orientation.

Cell Culture

Adenovirus transformed human embryonal kidney cells (293 cells) and SV40 transformed monkey kidney cells (COS cells) were grown in a modified minimal essential (MEM) and Dulbecco's modified Eagle's medium, respectively. Both media were supplemented with 10% fetal bovine serum, 4.4 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 250 ng/ml amphotericin B. Twenty-four hours before transfection, the cells were replated on 100-mm dishes at the same density (5×10^6). On the day of transfection fresh medium was added to the cells 4 h before transfection.

Transfection

All transfections were performed using the calcium phosphate precipitation method (Graham et al, 1973, Virology 52:456-467). The precipitate was applied for 4 h, the cells were washed, and fresh medium was added. Total RNA was isolated according to the method of Cathala et al, 1983, DNA 2:329-335. The pAV2 plasmids were transfected into both 293 and COS cells in Exp 1 and into only 293 cells in Exp 2. The pSV2.G plasmids were only transfected into COS cells. When either the α - or β -subunit was transfected alone into cells, 15 μ g purified plasmid were applied to each plate. When both the α - and β -subunit were cotransfected, 9 μ g each purified plasmid were applied to one plate. In some cases, cells were cotransfected with pVARNA. pVARNA consists of an adenovirus type 2 DNA HindIII B fragment containing the genes for VA_I and VA_{II} inserted into the HindIII site of pBR322 (obtained from Ketner, Department of Biology, Johns Hopkins University, Baltimore, MD). Va_I RNA stimulates translation by inhibiting phosphorylation and inactivation of the α subunit of eucaryotic initiation factor 2 (Akusjarvi et al, 1987, Mol Cell Biol 7:549-551).

RNA Analysis, RIA, and IRMA

Northern blot analysis of total RNA from transfections was performed using standard methods (Maniatis et al, 1982, Molecular Cloning, ed 1, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, p 202) and manufacturer's specifications. Common human α -subunit RIA, hTSH α -subunit RIA, and hTSH IRMA were performed in duplicate on the medium from each transfected culture (McBride et al, 1985, Clin Chem 31:1865-1867; Kourides et al, 1974, Endocrinology 94:1411-1421). The sensitivities of the assays were less than 0.03 ng/ml, less than 0.03 ng/ml, and less than 0.06 ng/ml, respectively. Cross-reactivity between the corresponding subunit and hTSH, at the measured concentration, was less than 5% in the common α and less than 2% in the hTSH β RIA (Kourides et al, supra). In addition, at the measured free subunit concentrations, the hTSH IRMA exhibited less than 1% cross-reactivity (data not shown).

Gel and Lectin Affinity Chromatography

The apparent mol wt of hTSH synthesized in 293 cells was determined by gel chromatography on a 1.5 x 90-cm Sephadex G-200 fine column calibrated during each chromatography run with five protein standards (bovine thyroglobulin, BSA, ovalbumin, bovine chymotrypsinogen A, and whale myoglobin). The column was equilibrated and run at 4°C in a buffer containing 0.12 M sodium chloride, 0.1 M borate, and 0.02% (wt/vol) sodium azide, pH 7.4. Two milliliters of fresh MEM medium containing 1000 μ U hTSH (WHO 80/558), 100 ng hCG α (CR-119), and 100 ng hTSH β (AFP-3929 β) were applied during chromatography of standard preparations. The column was washed and then 2 ml MEM medium from 293 cells transfected with pAV2-hCG or pAV2-hTSH β /pVARNA were applied. Fractions of 1.5 ml were collected at a flow rate of 6 ml/h.

The binding of hTSH synthesized in 293 cells to concanavalin A-Sepharose was also determined using methods previously described (Gesundheit et al, 1987, J Biol Chem 262:5197-5203). Samples were applied to lectin

column, and 2-ml fractions were collected at a flow rate of 10 ml/h. Human TSH IRMA was common human α - and hTSH β subunit RIAs were performed on fractions from gel and lectin chromatography. Recovery of hTSH and its free subunits was generally greater than 90% from chromatography.

TSH Bioassay

Thyrotropic bioactivity was measured as the ability to stimulate the uptake of ^{125}I into rat thyroid cells (FRTL5) in accordance with the procedure of Dahlberg et al, 1987, J Clin Invest 79:1388-1394. This assay measures human, rat, and bovine thyrotropin but not gonadotropins or free α - or TSH β -subunits. Sample determinations were performed in duplicate and compared to two pituitary hTSH standards (World Health Organization 80/558 and National Institutes of Health I-6). Results are expressed as microunits per ml; one microunit of WHO 80/558 is equivalent to 0.09 ng of NIH I-6 purified hTSH (unpublished data).

Statistics

Significant differences in immunoassay of cell media from various control and transfected cultures were determined using Student's t test.

RESULTS

Human TSH β Gene

A 17 kb genomic fragment was isolated by screening 1×10^6 recombinant phage clones. A restriction map (Fig. 1) was constructed using Southern blots of phage DNA hybridized with both a mouse TSH β cDNA probe (lacking 5'-untranslated sequences) and a 34 bp bovine 5'-untranslated sequence probe. Two coding exons are separated by a 450 bp intron and the sequence is identical to the published partial sequence (Hayashizaki et al, 1985, FEBS Lett 188:394-400) (data not shown). However, the complete coding sequence was not heretofore known.

Transfection

Two experiments were performed to compare the

level of mRNA and protein production between the most active adeno-associated virus promoter, P40, and the early promoter of SV40.

Table 1 shows the RIA and IRMA assay results from these two experiments. Interestingly, the 293 cells synthesized small amount of free α -subunit (control) whose levels were increased approximately 10-fold in a transfection with the calcium phosphate precipitate but without DNA (mock) ($P < 0.0005$). While neither transfection with pAV2-hCG α nor pVARNA increased α -production above the level of a mock transfection, the combination increased free α -levels 3- to 5-fold ($P < 0.0005$). Thus exogenous sources of the human α - subunit were clearly important in mediating this increase. The same pattern of pVARNA increasing protein production was seen when pAV2-hTSH β was transfected. 293 cells do not produce hTSH so that the medium of cells exposed to a mock or pVARNA transfection did not have measurable hTSH β . Only the 293 cells exposed to pAV2-hTSH β produced hTSH. When the plasmid was transfected alone the hTSH formed was due to combination with endogenous α . Cotransfection with both α - and β -plasmids, though, increased hTSH levels 1.5- to 2-fold ($P < 0.05$).

COS cells synthesized neither free α nor β but could synthesize hTSH β and hTSH when transfected with the appropriate plasmids. The levels of protein production were 10- to 100-fold less than in 293 cells and were only measurable when the pVARNA plasmid was also transfected. Regardless of whether pAV2 or PSV2.G was used, protein levels were barely if at all measurable without the pVARNA plasmid.

Figure 2 shows a Northern blot of total cellular RNA hybridized with the mouse TSH β cDNA probe. In 293 cells, hTSH β message was not detected from non-transfected (control) or from mock transfected cells. However, three RNA species of 2.3 kb, 1.6 kb, and 650 bases were noted after transfection of pAV2-hTSH β and

pVARNA (Lane 3). These three bands are the same size as those predicted from pAV2-hTSH β if transcription began at all three adeno-associated viral promoters (Fig. 2). The mRNA of 650 bases presumably represent a properly spliced hTSH β message. Lane 4 shows the same three bands but at lesser intensity when pAV2-hCG α , pAV2-hTSH β , and pVARNA were cotransfected. This reduction in signal intensity seen in lane 4 may have been due to the reduction in the amount of pAV2-hTSH β transfected from 15 μ to 9 μ g.

Control and mock transfected COS cells also did not contain hTSH β message. When pSV2.G-hTSH β was transfected (lanes 7-9), a major band of 900 bases and a minor band of 3.0 kb were seen. Without being bound to any theory, it is postulated that the 900 base species could represent a mRNA with the 277 bases of 5'-intron remaining, while the 3.0 kb species could represent read through of the hTSH β polyadenylation signal-site and use of the polyadenylation signal-site of pSV2.G (See Fig. 2).

Specific human α mRNA transcripts of appropriate size analogous to the hTSH β mRNA above were observed in cells transfected with pAV2-hCG α (data not shown). Since the main object of this invention is protein expression, the relative contribution of human mRNA from endogenous vs. exogenous (pAV2-hCG α) sources in 293 cells was not determined. However, the data suggest that the high level of free α -subunit observed after transfection with pAV2-hCG α /pVARNA is most likely due to mRNA from exogenous sources.

Gel and Lectin Affinity Chromatography

The apparent molecular weight of hTSH and its subunits synthesized in 293 cells after transfection with pAV2-hCG α /pAV2-hTSH β /pVARNA was determined on a G-200 Sephadex column (Fig. 3). In addition, standard preparations of hTSH, hCG α , and hTSH β were chromatographed on the same column. Internal protein standards had identical elution patterns between runs as determined by

optical density at 280 nm. In each case, the apparent mol wt of synthetic hTSH and its subunits was larger than its corresponding standard. Specifically, synthetic hTSH displayed an apparent mol wt of 45,000 and was larger than standard pituitary hTSH (apparent M_r = 40,000). This clearly indicates that the recombinant TSH is not constitutively identical to the natural product. The human α and hTSH β from transfection coeluted with the hTSH pituitary standard, and both were larger than their respective standard subunit preparation. However, in the case of free human α -subunit, the relative contribution to this chromatography pattern of endogenous α as compared to exogenous α from pAV2-hCG α cannot be determined.

The binding pattern to concanavalin A-Sepharose of synthetic hTSH from 293 cells as compared to standard human pituitary hTSH is shown in Table 2. Synthetic hTSH was glycosylated as indicated by complete binding to concanavalin A-Sepharose. The different elution pattern of standard vs. synthetic hTSH from the lectin columns is indicative of a difference at least in carbohydrate structure, again showing that the recombinant TSH (rTSH) is distinctly different from the naturally occurring TSH.

Immunoactivity and Bioactivity

Figure 4 shows that the hTSH produced in cell culture was indistinguishable from two pituitary hTSH standards in an assay involving two antibodies directed at different epitopes of the hTSH heterodimer (McBride et al, supra). The slopes were parallel over the entire range of values. Figure 5 shows the same hTSH in a ^{125}I trapping in vitro TSH bioassay compared to the same pituitary hTSH standards. The in vitro bioassay of standard pituitary hTSH or the cell culture product from 293 cells (pAV2-hCG α /pAV2-hTSH β /pVARNA) was normalized to immunoreactivity in a hTSH immunoradiometric assay (IRMA) assay. The dose response and ED_{50} of the standards and cell culture product were identical. In addition, the cell culture product from COS cells (pAV2-hCG α /pSV2 β

/pVARNA) was biologically active although the lower level of expression prevented determination of a dose response curve.

5 In summary, a 17 kb genomic fragment of hTSH β has been isolated and both coding exons of this gene produced hTSH β and hTSH in a transient expression assay. This is the first report of TSH from any species produced by gene transfection in cell culture. The expression vectors of hTSH β included only the two coding
10 exons, and not the 5'-untranslated exon of the gene (Wondisford et al, Mol. Endo. 2(1); 32-39, 1988).

Transient expression after gene transfection was used to test both the early promoter of SV40 or the P40 promoter of adeno-associated virus. The early pro-
15 moter in COS cells produced more mRNA than the P40 promoter in 293 cells regardless of whether pVARNA was cotransfected. However, pVARNA clearly increased mRNA levels in either vector system. This suggests that in addition to increasing the rate of translation, pVARNA
20 must either increase transcriptional rate, RNA transport, or stability.

While the pSV2.G-hTSH β expressin vector produced higher levels of hTSH β mRNA than pAV2-hTSH β , this mRNA was about 250 bases larger than that found in the
25 human pituitary. The 450 bp intron was certainly spliced out since this intron in the mature message would have prevented hTSH β protein synthesis. Also, an mRNA of appropriate size was produced by PAV2-hTSH β indicating that the polyadenylation site in the fragment must be
30 active. Thus, the most likely reason for a larger hTSH β mRNA in COS cells was the lack of splicing of a 277 bp intron fragment upstream of the first coding exon. Eighteen base pairs downstream of the transcriptional start site of the P40 promoter is a consensus splice
35 donor site which could explain why the 277 pb intron fragment would be spliced out in the adeno-associated virus vector.

The plasmid, pVARNA, increased protein production in either vector system, but the P40 promoter in 293 cells led to expression of between 10- to 100-fold more protein than the early promoter of SV40 when cotransfected with pVARNA. This is most likely due to an increased translational rate mediated by pVARNA as has been previously demonstrated for expression of other mRNAs (Akusjarvi et al, 1987, Mol Cell Biol 7:549-551). Of course, the possibility that the larger mRNA from pSV2.G-hTSH β contributed to the lower protein levels from COS cells cannot be excluded.

The hTSH produced in cell culture was functionally indistinguishable from two pituitary hTSH standards in both a highly specific IRMA and in vitro bioassay. It should be noted, however, that the synthetic hTSH of the present invention was larger in size than standard pituitary hTSH on gel chromatography. Although it was glycosylated as indicated by complete binding to concanavalin A, it displayed a somewhat different pattern on lectin chromatography as compared to a standard hTSH preparation. The larger mol wt of these synthetic glycoproteins as compared to pituitary standards is most likely due to an altered glycosylation pattern such as more sialylation. In the case of hTSH, this might also reflect a β -subunit containing the 118 amino acids predicted from the nucleic acid sequence rather than the 112 found in standard hTSH purified from postmortem human pituitaries.

Transient expression is more convenient than stable integration in the analysis of a large number of expression vectors. pAV2 and pVARNA now allow transient expression of hTSH in 293 cells at levels high enough to analyze protein and glycosylation site structure-function relationships. Previously, the only information about such relationships came from studies involving chemical modifications of protein by iodination, nitration, acetylation, and carboxymethylation (Pierce et al, 1981,

Annu Rev. Biochem., Annual Reviews Inc., Palo Alto, CA, pp 465-495) or inhibition of glycosylation by tunicamycin (Weintraub, et al, 1980, J Biol Chem 255:5715-5723). The chemical groups could themselves change protein conformation irrespective of the alteration in amino acids they produce and inhibition of glycosylation affects not only TSH but all cellular glycoproteins. Site-directed mutagenesis of the hCG α cDNA and human TSH β -gene could directly address what regions are important for protein conformation, subunit combination, receptor binding, biological activity, and metabolic clearance without introducing chemical groups or unknown changes into the protein structure.

The availability of substantially pure rTSH now makes the diagnosis and treatment of human thyroid cancer and the determination of the level of TSH a reality.

Currently, the only available method to diagnose and treat human thyroid cancer involves making patients hypothyroid and allowing their own endogenous human TSH to rise after several weeks to stimulate the uptake of ^{131}I into the cancer. Such stimulation is used as a diagnostic test to localize the tumor by scanning and is subsequently used to treat the cancer by giving large doses of ^{131}I . All of the diagnostic tests and therapies depend on high levels of human TSH. However, the technique of producing endogenous hypothyroidism has disabling side effects including lethargy, weakness, cardiac failure, and may also lead to a rapid growth of the tumor over the several week period of treatment. In contrast, if a desirable form of synthetic human TSH were available, patients could be treated while they were euthyroid by giving exogenous injection of the TSH. However, presently it is not feasible to give exogenous TSH because there is not enough natural product from available human pituitaries collected at autopsies. Furthermore, even if available, the human pituitaries have been found to be contaminated with viruses and the

National Pituitary Agency has forbidden the use of the natural product for any human diagnostic or therapeutic studies. This is true for all human pituitary hormones including human growth hormone which is now exclusively
5 marketed as a synthetic product. However, the technology that was applicable for human growth hormone (a non-glycoprotein) is not at all applicable for human TSH (a glycoprotein hormone of two glycosylated subunits). As has been described herein supra, only the methodology
10 described herein relating to transfection and proper glycosylation of each subunit in mammalian cells produces a desirable biologically active rTSH material. Moreover, it has been found that the altered glycosylation pattern that can be achieved with the cells, as described in the
15 methodology of the present invention, produces a longer acting human thyrotropin which is particularly suited for the diagnosis and treatment of thyroid cancer.

The diagnosis and treatment of thyroid cancer involves first purifying the synthetic TSH from large
20 volumes of tissue culture media harvested from approximately ten billion cells over two to four weeks. Using a chemically defined medium to reduce protein contaminants as is well known in the art, synthetic human TSH, which represents about five to ten percent of all the protein
25 secreted into the medium, can be obtained. The human TSH thus obtained is then purified by a combination of standard techniques including immunoaffinity chromatography, HPLC exclusion chromatography (repeated two to three times) followed by dialysis and concentration by
30 ultrafiltration, lyophilization and the like. The purified human TSH is then tested in animals to assure its efficacy as well as freedom from any unexpected toxicity. The synthetic TSH is then tested in patients in clinical trials using different doses to determine the
35 optimal doses to achieve maximal uptake into the tumor for both diagnosis and treatment with ^{131}I . During initial try-outs for diagnosis, one to two administra-

tions of about 100 μ g, while during therapy three to six doses of about 100 to 200 μ g may be administered, but the optimal dose schedule is determined by the results of the clinical trials. It is noted that all of these procedures are accomplished while the patient is still euthyroid without producing any of the disabling side effects of hypothyroidism which are otherwise encountered in the methods heretofore available.

When the optimal uptake of ^{131}I has been established, patients may be treated with doses of about 50 to 400 mCi of ^{131}I and the effect of therapy assessed by subsequent ^{131}I diagnostic tests as well as conventional x-rays, CAT scans, measurement of serum thyroglobulin and the like. Of course, ^{131}I -labeled rTSH, which is produced by standard methodology well known in the art, can be appropriately utilized in the procedures mentioned above.

It is estimated that there will occur about ten thousand new cases of thyroid cancer in the United States each year and a very large prevalence of older cases of this cancer require repeated diagnostic and therapeutic intervention which are currently unsatisfactory. Availability of synthetic human TSH as taught herein, even at a cost of \$50.00 to \$100.00 per injection, will still be a relatively inexpensive part of the complete evaluation and therapy for this difficult, but curable cancer.

Another advantage of the product of the present invention is to provide assay components for human thyrotropin using the technique of radioimmunoassay. Certain immunoassay kits are presently available, but the reagents therein are again derived from a very short supply of natural product. Moreover, the natural product varies greatly depending on the source of the human pituitaries as well as the degree of degradation that occurs during autopsy. This has led to considerable variation among commercially available kits with disagreements in results of the TSH testing among various

kits. In contrast, the present invention, for the first time, provides a virtually unlimited supply of a stable preparation of synthetic TSH allowing kit manufacturers to have a universal standard preparation and a virtually identical and inexhaustible supply of the reagents. This would allow world wide consistency of dosage and lead to much needed standardization in the measurement of human TSH which is vital in the assessment of thyroid function in humans. This is accomplished by labeling the rTSH with radioactive iodine (I^{131} , I^{125}) or another suitable labeling material such as chemoluminescent or fluorescent labels and by producing antibodies to the pure product by either polyclonal or monoclonal techniques which are well known in the art and providing inexhaustible supplies of immunoglobulins without significant interfering cross-reactivity with other hormones. The antibodies are then formulated in classic radioimmunoassay kits which are supplied to the manufacturers to be used in a variety of standard assay methodologies (RIA, IRMA, Sandwich Assays and the like).

There are various other advantages of rTSH. Tests have demonstrated that it is possible to modify the TSH by expressing the hormone in various cell lines leading to altered glycosylation patterns. Moreover, using the technique of site-directed mutagenesis whereby individual bases in the DNA are changed, products are obtained with altered biologic function such as prolonged or decreased half life, as well as competitive antagonists that bind to the TSH receptor and actually block TSH function. Such competitive antagonists are useful in a novel way to treat diseases such as TSH-induced by perthyroidism as well as Graves' disease which is caused by auto-antibodies to the TSH receptor. The function of these abnormal stimulators would be blocked by the competitive antagonist that we have already shown to be active at the cellular level. Moreover, using various long-acting and short-acting preparations, superagonists

can be prepared which would be particularly stimulating to thyroid function, and superantagonists can be prepared which would be particularly inhibitory of thyroid function. In this manner, thyroid function can be controlled in many different types of disease of thyroid overactivity or underactivity. It should be noted that these completely novel approaches are feasible only because of the availability of the synthetic TSH by the methodology of the present invention because, for the reasons mentioned above, the natural product is prohibited from such in vivo use.

It has also been discovered that modifications of the transfection process greatly enhances the amount of TSH production by mammalian cells. For example, instead of using TSH- β gene constructs containing only the 2nd and 3rd exons (the 2 coding exons), a new construct is made by adding the first untranslated exon of TSH- β . The inclusion of this untranslated TSH- β exon greatly increases TSH production. Without being bound to any specific theory, it is postulated that the enhanced TSH production occurs by increased transcription rate and/or mRNA stability. Moreover, it has been discovered that an excess of the α gene in a ratio of about 3 to 5 times greater than the β gene, yields high rate of TSH production (10-50 mg/month), close to commercial scale production.

A standard concentration curve utilizing anti-TSH antibodies is established to determine the amount of TSH in a sample by conventional immunological assays.

In summary, a recombinantly made synthetic TSH has been made which, even though constitutively distinct from the natural product, possesses functional properties similar to the natural product and is useful for diagnostic as well as therapeutic purposes.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light there-

of will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

Table 1. Immunoassay for Human TSH and its Subunits in Cell Media from Control and Transfected Cultures

	Transfected Construct or Control (Exp no.)	Human α RIA (ng/ml)		Human TSH RIA (ng/ml)		Human TSH IRMA (U/ml)	
		n	Mean	SEM	Mean	SEM	Mean
5	293 cells						
	Medium (1,2)						
	Control (2)	4	<0.03		<0.03		<0.6
	Mock (2)	4	0.31	0.01 ^a	<0.03		<0.6
10	pVARNA(2)	4	3.4	0.36 ^b	<0.03		<0.6
	pAV2(2)	4	5.0	0.68	<0.03		<0.6
	pAV2 α (2)	4	2.1	0.11	<0.03		<0.6
	pAV2 α /pVARNA(2)	4	1.6	0.31	<0.03		<0.6
15	pAV2 β (2)	4	17.3	1.0	<0.03		<0.6
	pAV2 β /pVARNA(1)	4	3.4	0.37	<0.03		<0.6
	pAV2 β /pVARNA(2)	2	2.2	0.30	1.5	0.09	2.8
	pAV2 α /pVARNA(1)	4	5.7	0.64	3.3	0.33	6.9
20	pAV2 α /pVARNA(1)	2	4.0	1.5	0.6	0.24	11.2
	pAV2 α /pVARNA(2)	4	6.1	0.54	1.1	0.09 ^d	15.1
	Medium (1,2)						0.30
	Control (2)						0.64 ^a
25	Mock (2)	2	<0.03		<0.03		6.3
	pAV2 α /pVARNA(1)	2	0.06	0 ^b	<0.03		3.6
	pSV2 β (1)	2	<0.03		<0.03	0 ^d	
	pSV2 β /pVARNA(2)	2	<0.03		<0.03		
	pSV2 α /pVARNA(2)	2	<0.03		0.16	0.01 ^d	
	pSV2 α /pVARNA(1)	2	<0.03		<0.03		
	pSV2 α /pVARNA(2)	2	0.09	0.02 ^b	0.10	0.02 ^d	0.06 ^c
	pSV2 α /pVARNA(1)	2					

Table 1 (continued)

Various constructs were transfected into either 293 or COS cells in two separate experiments. The medium was harvested after 2 days in Exp 1 and 3 days in Exp 2. Cell medium was assayed for the human α -subunit, hTSH β subunit, and hTSH as shown. Human CG α and hTSH β were labeled α and β in construct names. n, Number of plates transfected, IRMA, 1 μ U is Immunologically equivalent to 0.09 ng NIH 1-6 purified hTSH. Medium, Fresh medium before application to cells. Control, Medium from nontransfected cells. Mock, Medium from cells transfected with a calcium phosphate precipitate lacking DNA.

ap < 0.0005 compared to mock, 293 cells.

bp < 0.0005 compared to pAV2 α /pVARNA(2), 293 cells.

cp < 0.05 compared to pAV2 α / β /pVARNA(2), 293 cells.

dp < 0.005 compared to pAV2 β /pVARNA(2), 293 cells.

Table 2. Lectin Chromatography of Synthetic and Standard hTSH

Column		Unbound (%)	Bound-		
			MG (%)	Bound-MM	
5	1	WHO STD	0	12	88
	2	pAV2 α / β /pVARNA	0	28	72

10 Binding of a synthetic vs. a standard hTSH preparation, described in the legend to Fig. 4, to concanavalin A-Sepharose, is shown. Results are expressed as a percentage of the total hTSH Immuno-activity eluted from two identical columns. Unbound, Bound-MG, Bound-MM = immunoactivity measured in 2-ml column fractions after elution with Tris-buffered saline, α -methylglucoside (10 mM), and α -methylmannoside (500 mM), respectively.

WHAT IS CLAIMED IS:

1. Substantially pure, biologically active recombinant human thyrotropin (rTSH).

2. The thyrotropin of claim 1 labeled isotopically with I^{131} , I^{125} , chemoluminiscently or fluorescently.

3. The thyrotropin of claim 1 produced by recombinant genetic process using constructs with gene elements that enhance thyrotropin production.

4. A clone comprising complete nucleotide sequence for the expression of the thyrotropin of claim 1 in a suitable expression vector.

5. The clone of claim 4 further comprising first untranslated exon of TSH- β .

6. A method for producing TSH, comprising
(a) allowing expression of TSH by the clone of claim 4 in a suitable expression vector; and
(b) then recovering substantially pure TSH by conventional purification and isolation methodology.

7. A method for producing TSH, comprising
(a) allowing expression of TSH by the clone of claim 5 in a suitable expression vector; and
(b) then recovering substantially pure TSH by conventional purification and isolation methodology.

8. A method for producing TSH, comprising
(a) allowing expression of TSH by the clone of claim 4, wherein TSH α is about 3 to 5 times in excess of TSH β ;

(b) then recovering substantially pure TSH by conventional purification and isolation methodology.

9. A method for producing TSH, comprising
(a) allowing expression of TSH by the clone of claim 5, wherein TSH α is about 3 to 5 times in excess of TSH β ;

(b) then recovering substantially pure TSH by conventional purification and isolation methodology.

10. A TSH antagonist produced by a mutant of

the clone of claim 4.

11. A TSH agonist produced by a mutant of the clone of claim 4.

12. A kit comprising a saleable package including containers separately containing

- (a) universal standard of substantially pure unlabeled rTSH;
- (b) substantially pure, labeled rTSH;
- (c) antibodies against purified rTSH; and
- (d) instructional material describing the use of reagents (a), (b) and (c).

13. Anti-rTSH antibodies without interfering cross-reactivity with non-TSH hormones.

14. A method for determining the level of TSH in a sample, comprising reacting an aliquot of a sample in which the amount of TSH is to be determined with the antibodies of claim 13 and comparing the level of antibody reactivity with a predetermined standard antibody-rTSH reactivity curve to determine the amount of TSH present in said sample.

15. A method of diagnosing the extent of thyroid cancer, comprising administering rTSH of claim 1 to a patient to maximize ^{131}I uptake, and then administering a visualizing dose of ^{131}I to said patient; and then visualizing the cancer by standard visualizing means.

16. A method for treating thyroid cancer, comprising administering a therapeutic regimen of a combination of rTSH of claim 1 and ^{131}I or ^{131}I -labeled rTSH to a patient afflicted with thyroid cancer.

17. A method of blocking TSH activity, comprising inhibiting TSH activity by competitive amount of the antagonist of claim 10.

18. A method of stimulating TSH activity, comprising inducing TSH production by the agonist of claim 11.

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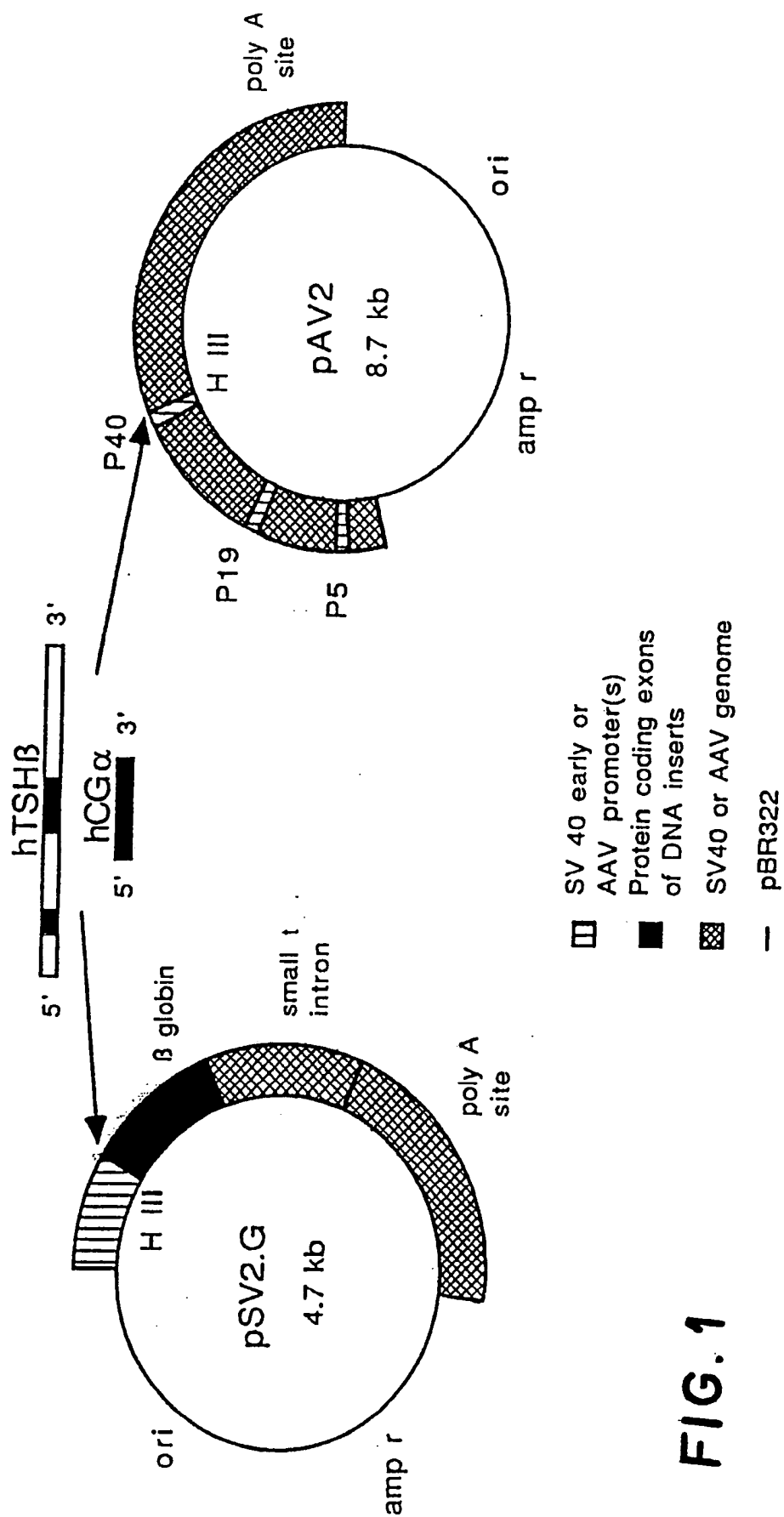


FIG. 1

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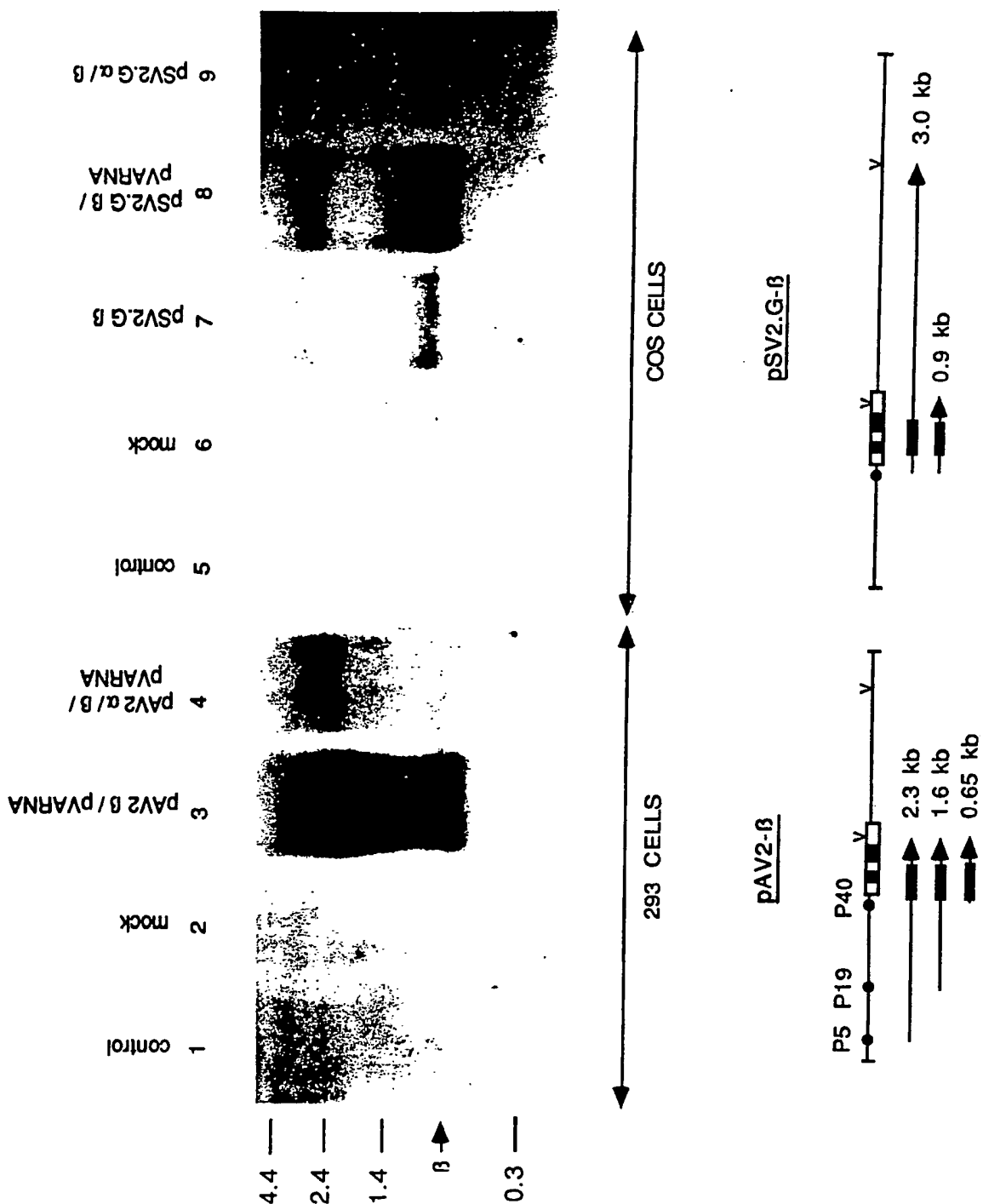
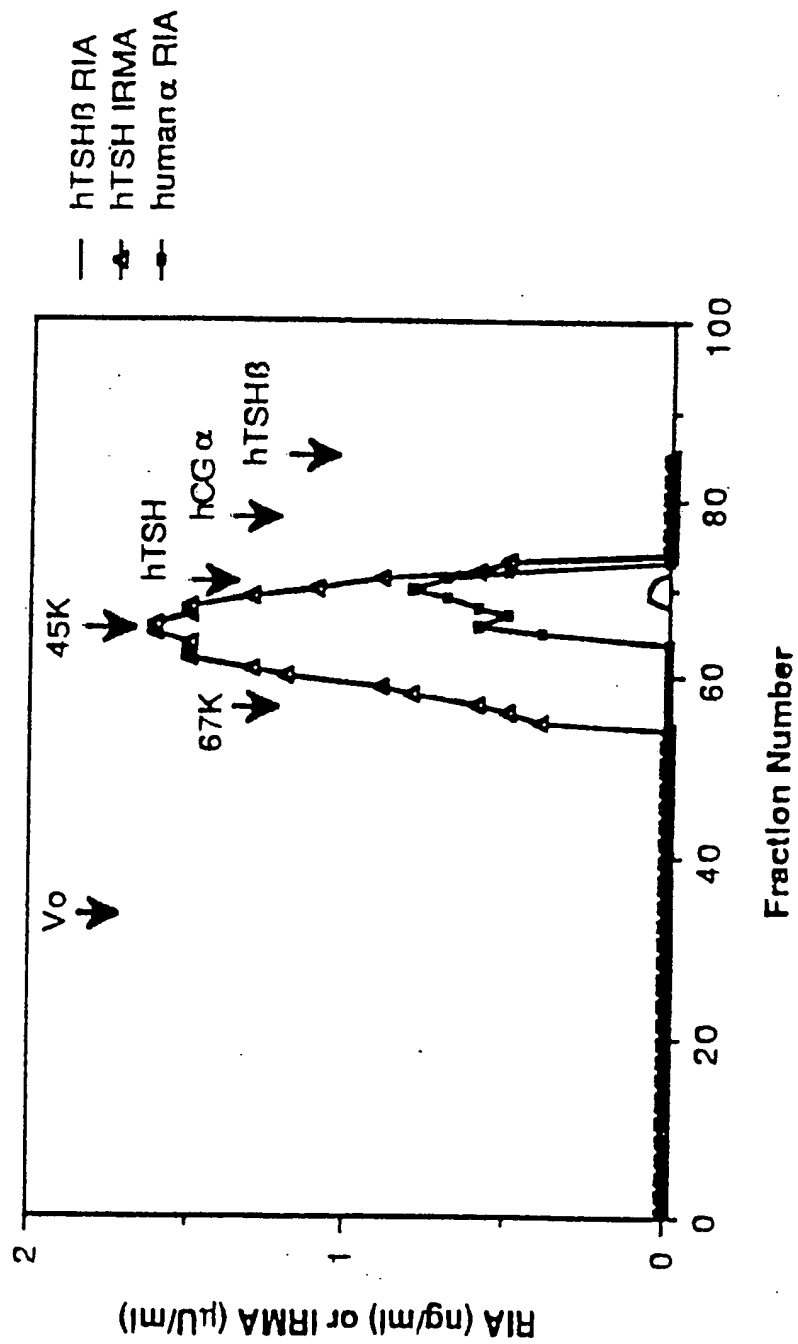


FIG. 2

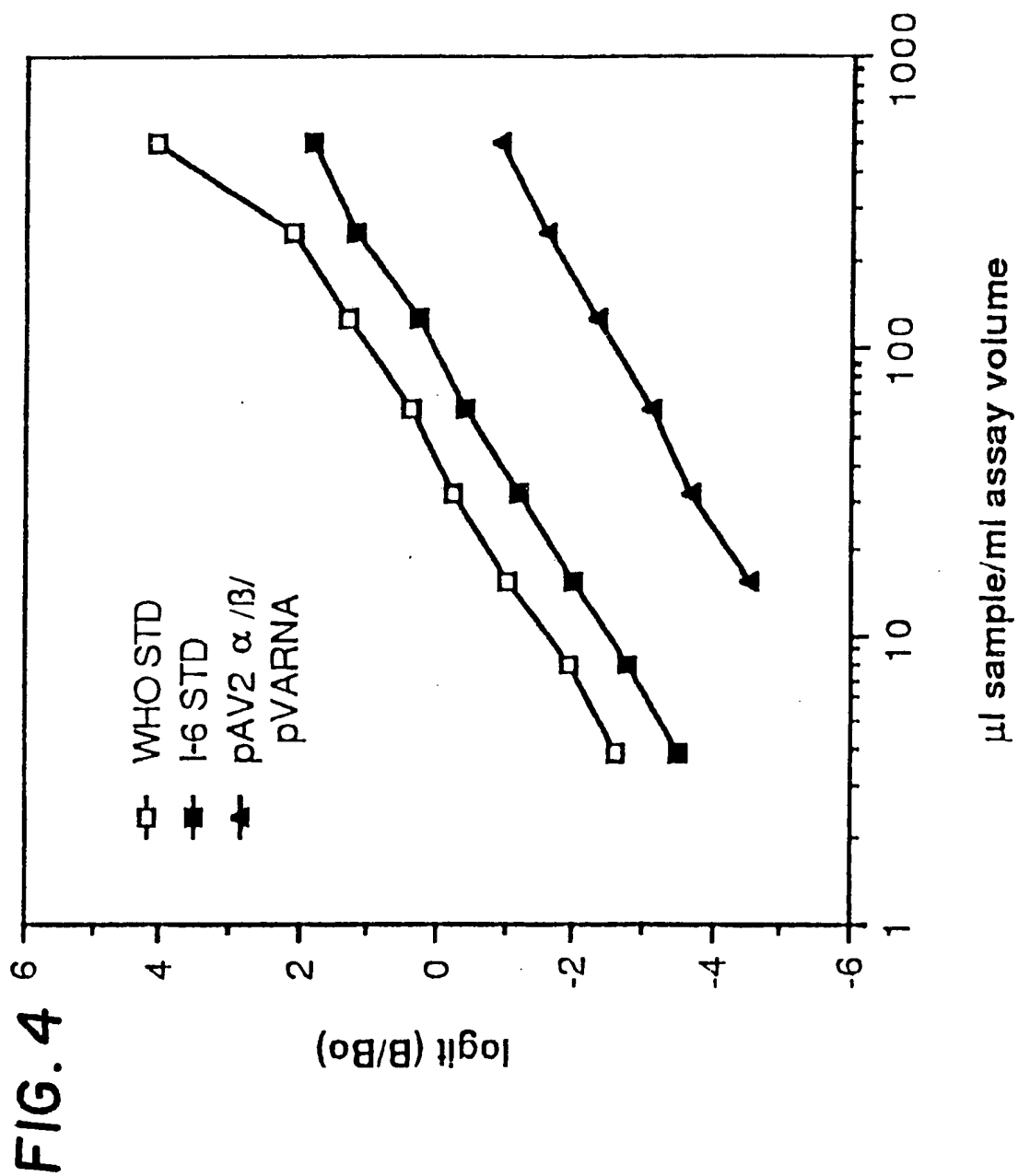
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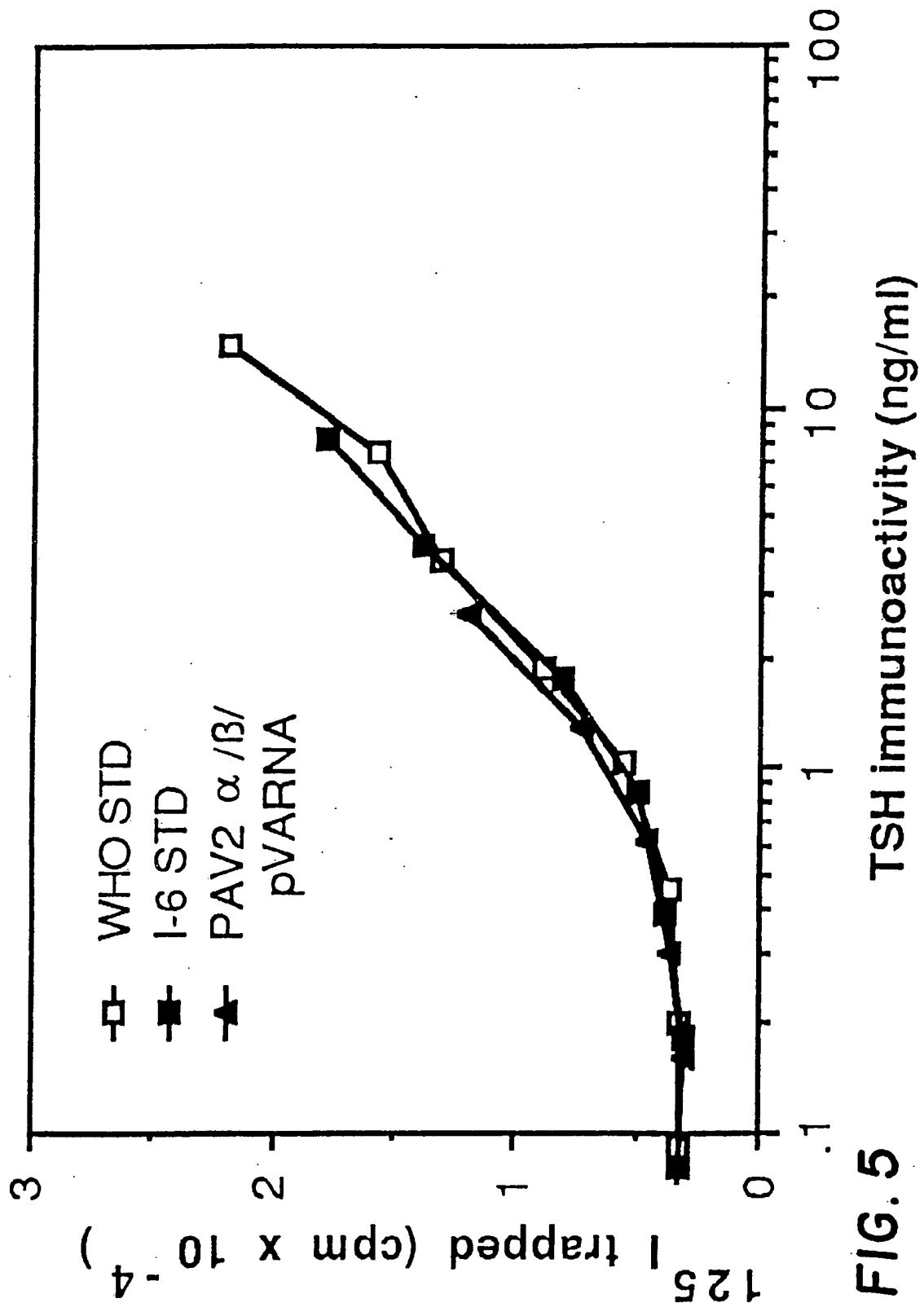
Gel Chromatography

FIG. 3

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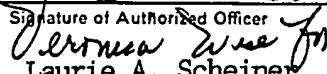


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INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/00115

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC (5): A61K 37/24; C12N 15/16; A61K 49/00 U.S. Cl: 530/397; 435/172.3; 424/9		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
U.S.	530/397; 435/172.3; 424/9	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
Chemical Abstracts Services Online (File CA, 1967-1989; File Biosis Previews 1969-1988)		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
<u>X</u>	EP, A, 0 228,604 (IONE KOURIDES et al.)	1, 3-5
<u>Y</u>	15 JULY 1987 (See abstract)	2, 6-11, 15
<u>X</u>	Chemical Abstracts, volume 95, No. 13, issued	1, 3-5
<u>Y</u>	28 SEPTEMBER 1981 (Columbus, Ohio, USA) John C. Fiddes, et al, " The Gene Encoding the common alpha subunit of the four glycoprotein hormones". See page 234, column 1, the abstract No. 110370q, J. Mol. Appl. Genet. 1981, 1(1), 3-18 (Eng).	2, 6-11, 15
<u>X</u>	Chemical Abstracts, volume 108, No. 17, issued	1, 3-5
<u>Y</u>	25 April 1988 (Columbus, Ohio, USA) Fredric E. Wondisford, et al. "Cloning of the human thyrotropin beta-subunit gene and transient expression of biologically active human thyrotropin after gene transfection", See page 187, column 2, the abstract No. 144501K, Mol. Endocrinal 1988, 2(1), 32-9 (Eng).	2, 6-11, 15
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
19 June 1990		<div style="font-size: 1.2em; font-weight: bold;">17 JUL 1990</div>
International Searching Authority		Signature of Authorized Officer
ISA/US		 Laurie A. Scheiner

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET**V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹**

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

- I. Claims 1-11 and 15 drawn to a protein classified in Class 530, Subclass 397.
- II. Claim 13 drawn to an antibody classified in Class 530, Subclass 387.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims: _____
3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers: **1-11 and 15**
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

continuation of VI. OBSERVATIONS OF INVENTION LACKING

- III. Claims 12 and 14 drawn to a kit and method of use classified in Class 435, Subclass 7.
- IV. Claim 16 drawn to a method of treating classified in Class 424, Subclass 1.1.
- V. Claims 17 and 18 drawn to methods of regulating TSH classified in Class 436 Subclass 500.

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